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Influence of Crystallization Habit of Minerals on 'In Vitro' Cytotoxicity

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16. ABSTRACT

Four samples of cummingtonite-grunerite series in various crystallization habits were tested in vitro. The cytotoxicity to Chinese hamster ovary cells and hemolysis to sheep erythrocytes was inversely proportional to the structural faults and surface defects of the minerals. At a comparable surface area, asbestiform grunerite (UICC amosite), semi-asbestiform cummingtonite, acicular cummingtonite, and acicular grunerite were found to be cytotoxic and hemolytic in a decreasing order. The influence of particle size on hemolysis and cytotoxicity was observed with acicular grunerite. Although samples of relatively large particle size were found to be inert, samples of smaller particle size were cytotoxic as well as hemolytic. No apparent relationship between surface charge and hemolysis as well as cytotoxicity was observed.

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INFLUENCE OF CRYSTALLIZATION HABIT OF MINERALS ON *IN VITRO* CYTOTOXICITY

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The health hazards caused by exposure to commercial asbestos particles are well known. The question, however, still remains whether the nonasbestiform equivalents of these minerals to which people may be exposed in mining, quarrying, and other activities can also have adverse effects on human health. Identification of minerals in various crystallization habits and their interactions with biological systems is pertinent. Unfortunately, direct study of the physical, chemical, and biological properties of all minerals and their varieties would be an extremely expensive and time-consuming project. A more practical approach is to define the physical and chemical properties of minerals and their varieties in different categories and test them simultaneously in selected biological systems. It is conceivable that extrapolations can be made to relate specific mineralogical properties to specific biological activities.

Several attempts have been made by other investigators to relate physical and/or chemical properties of minerals with biological properties. While some studies revealed that hemolysis of mammalian erythrocyte is related to mineral surface area,[1,2] others have shown that it depends on surface charge,[3,4] and yet others have indicated a relationship to magnesium content.[1,5,6] In an earlier study, we demonstrated that still another mineral characteristic—namely, asbestiform crystallization habit—is responsible for sheep erythrocyte hemolysis and rabbit alveolar macrophage cytotoxicity.[7]

The study is designed in an attempt to define the roles of physical and chemical characteristics in the biological system. Four samples of cummingtonite-grunerite series in different crystallization habits are selected. In preparation for biological studies, these minerals are ground to obtain samples of various particle sizes. All samples are characterized for chemical composition, size distribution, and surface charge. Biological activity of the samples is assessed in terms of cell lysis of sheep erythrocytes and cytotoxicity to Chinese hamster ovary cells.

*This report presents the results of work performed by Northrop Services, Inc. (under Contract Number 68-02-2566) and GCA Technology (under Contract Number 68-02-2771) for the United States Environmental Protection Agency.

Materials and Methods

Sample Selection

Asbestiform Grunerite

This is the classical South African asbestiform grunerite; the actual sample used is the UICC amosite standard.

Acicular Grunerite

This sample is obtained from Smallwood Mine (Wabush, Labrador, Canada) and is composed of parallel acicular crystals. The color of the semitranslucent crystals is yellowish green and their luster is subadamantine and silky.

Semiasbestiform Cummingtonite

This sample is obtained from Homestake Mine (South Dakota, U.S.A.). The crystals display a definitely fibrous appearance, but are less conspicuously asbestiform than amosite. The color of the semitranslucent crystals is greenish brown and their luster is adamantine and pearly, with some portions showing the silky luster of amosite. The length of the crystals ranges from a few millimeters to almost 10 cm; the width appears to be submicroscopic. The fibers can be separated by a needle with some difficulty (that is, not as easily as amosite fibers). The fibers are stiff in larger dimensions and become flexible as the size of the fibers is decreased. None of the fibers show the characteristic silky shine and high flexibility of amosite.

The term "semiasbestiform" is introduced to express this sample's intermediate asbestiform characteristics. Unfortunately, no quantitative expression is available at present to express the degree of asbestiform development of amphibole fibers. All that can be stated at this point is that the development of asbestos properties in this sample is somewhere between the lack of asbestos properties of the acicular variety and the highly developed asbestos properties of amosite.

Acicular Cummingtonite

This sample is obtained from Homestake Mine (South Dakota, U.S.A.) and is composed of radiating acicular crystals. The color of the opaque-to-semitranslucent crystals is similar to the semiasbestiform crystals but darker, and the luster is more dull than that of the semiasbestiform fibers. The length of the crystals ranges from 0.5 to 10 mm; the width is 0.1 mm or less. Most of the crystals are stiff and brittle; some, however, demonstrate a small degree of flexibility. These latter crystals are removed from the sample when noticed; however some are probably missed in this crude visual process. The sample is expected to have a small degree of asbestiform character.

Sample Analysis

Chemical Analysis

Electron probe microanalysis is performed with a MAC Model 5 probe. Approximately 10 mg of sample are mounted in the epoxy medium and the sample is viewed

optically in the instrument at a beam size of 5 μm in diameter. Silicate and oxide standards are used in calibration of the probe. The instrument is computerized and matrix corrections are made using the Bence-Albee recursive method. Approximately six analyses are made for several particles ranging from 10 to 20 μm in diameter. The average of these point analyses is generally within ± 5 percent, where there is no chemical zoning.

Measurement of Surface Area

Surface area is determined by the BET adsorption isotherm method using N_2 as the adsorbent. Determinations are made in an all-glass vacuum system operating at a pressure of less than 2×10^{-6} torr. An oxygen vapor thermometer is used to measure directly the partial pressure of nitrogen in the liquid nitrogen bath and hence that equilibrium value inside the sample bulb. This method gives a more direct, accurate value than would be obtained with a thermocouple.

Measurement of Zeta Potential

The surface charge of gross dispersion of the minerals in buffered solution is measured using a microelectrophoresis instrument. The instrument cell attached to the microscope is filled with 25 ml of freshly prepared suspension of particles. This, in turn, completes a circuit giving a potential difference of about 150 V across the cell. Measurements are by means of a null technique (stopping the "cloud" of particles which appear to move across the viewing field), rather than the commonly used timing of transit of an individual grain across a known distance.

Assessment of Hemolysis of Sheep Erythrocytes

The sheep blood is obtained in Elsevier's solution commercially. Before use, the erythrocytes are washed three times in veronal-buffered saline and a standard 2 percent suspension is prepared. As a standard reference, a lysate is prepared by adding 1 ml of 2 percent erythrocyte suspension to 3 ml of water. To standardize the Varian spectrophotometer, the lysate is read at 540 mM. Proper adjustments are made if the lysate absorbance is not between 0.700 and 0.750.

The test samples at a known concentration are added to a test tube containing 3 ml of Veronal-buffered saline and incubated at 37°C for 10 minutes and then 2 percent erythrocyte suspension is added. The tubes are then incubated at 37°C for 50 minutes. After this period, the tubes are centrifuged at 1500 rpm and the supernatant is read on the Varian spectrophotometer at 540 mM and percent hemolysis is calculated as follows:

$$\frac{\text{Optical Density of Sample}}{\text{Optical Density of Lysate}} \times 100 = \% \text{ Hemolysis}$$

Each sample is checked for surface adsorption of hemoglobin. Four ml of lysate are added to a known amount of test sample and incubated for 50 minutes at 37°C. The tubes are then centrifuged and the supernatant is read on the Varian spectrophotometer at 540 mM and percent adsorption is determined as follows:

$$\frac{\text{Optical Density of Lysate} - \text{Optical Density of Sample}}{\text{Optical Density of Lysate}} = \% \text{ Adsorption}$$

If a particulate sample is found to adsorb hemoglobin, the values of percent adsorption and hemoglobin are added to obtain actual hemolysis.

Assessment of Cytotoxicity to Chinese Hamster Ovary (CHO) Cells

The CHO cell line is obtained from the American Type Cell Collection. The cells are maintained in F-12 medium supplemented with 10 percent fetal calf serum, 100 units penicillin/ml and 100 μg/ml streptomycin. The cultures are incubated at 37°C and gassed with 5 percent CO_2 in air.

The cultures are seeded at 500 cells/25-mm^2 Corning flask in 4 ml of the nutrient medium and incubated at 37°C for 24 hours for attachment. After this period, a known amount of test sample is added to the cultures and incubated for 6 days; during this time, the cells divide and form separate colonies. The medium is then removed, and a mixture of 0.5 percent NaCl and 4 percent methanol in 10 percent formalin is added to fix the colonies. The colonies are stained with 0.04 percent crystal violet and counted (using a colony counter). The number of colonies is determined as a percent of control (cells without test particles).

Results

Hemolysis

Chemical analysis revealed that all minerals are iron-rich silicates (Table 1). The Fe/Mg ratios of 80/20, 90/10, 70/30, and 70/30 confirmed that the first two samples, UICC amosite and Labrador grunerite, are indeed grunerites and that the other two samples, semiasbestiform and acicular cummingtonite, are truly cummingtonites.

A comparison of hemolysis caused by these samples is indicated in Table 2. The degree of hemolysis caused by the asbestiform variety (UICC amosite) was much higher than that caused by other varieties of comparable surface area. Asbestiform particles with a surface area of 4.13 m^2/g caused 53.3 percent hemolysis, whereas

Table 1
Electron Probe Microanalysis

	Cummingtonite–Grunerite			
Elements wt. % Oxide	Asbestiform Grunerite %	Acicular Grunerite %	Semiasbestiform Cummingtonite %	Acicular Cummingtonite %
SiO_2	43.13	48.34	52.36	52.77
FeO	29.95	43.93	33.76	34.02
MgO	3.50	3.04	8.10	8.16
Na_2O	0.21	0.03	—	0.40
K_2O	1.87	0.05	—	—
CaO	0.24	0.31	0.94	0.95
MnO	1.04	0.70	0.45	0.45
TiO_2	0.27	0.03	—	—
Al_2O_3	5.43	0.36	1.54	1.55
Fe/Mg	80/20	90/10	70/30	70/30

TABLE 2
HEMOLYSIS CAUSED BY CUMMINGTONITE-GRUNERITE MINERALS

Mineral	Hemolysis at 20 mg/ml %	Surface Area m^2/gm	Surface Charge mV
Asbestiform Grunerite (UICC Amosite)	53.3 ± 4.5	4.13	−26.9 ± 3.1
Semiasbestiform Cummingtonite	32.48 ± 7.69	3.88	−22.5 ± 2.2
	31.8 ± 7.19	1.61	
	32.3 ± 4.78	1.21	
Acicular Cummingtonite	29.57 ± 7.43	3.76	
	31.4 ± 4.2	2.45	−15.7 ± 2.4
	18.94 ± 2.52	.82	
Acicular Grunerite	40.0 ± 7.1		
	32.48 ± 4.6		
	15.83 ± 2.0		−26.2 ± 4.0
	10.0 ± 1.0		
	0	2.82	

acicular particles with a surface area of 2.82 m^2/g caused no hemolysis. The in-between samples, semiasbestiform and acicular cummingtonite, were found to be less hemolytic than the asbestiform grunerite but more hemolytic than the acicular grunerite. Semiasbestiform cummingtonite samples of surface area 3.88 m^2/g caused 32.48 percent hemolysis, whereas acicular cummingtonite samples of surface area 3.76 m^2/g caused 29.57 percent hemolysis. Although of different crystallization habit, these two samples did not exhibit any difference in hemolytic activity. The semiasbestiform samples of surface area 3.88 m^2/g and 1.61 m^2/g caused 32.48 percent and 31.8 percent hemolysis, respectively. The acicular samples of surface area 3.76 m^2/g and 2.45 m^2/g caused 29.57 percent and 31.4 percent hemolysis, respectively. At a lower surface area, however, the samples showed a significant difference in hemolysis. Semiasbestiform variety of surface area 1.21 m^2/g caused 32.2 percent hemolysis, whereas acicular samples of surface area 0.82 m^2/g caused only 18.94 percent hemolysis.

A relationship between the degree of hemolysis and particle size of the samples was demonstrated with acicular grunerite samples of a variety of size distribution. Although the sample of 2.82 m^2/g surface area caused no hemolysis, when the sample was ground further, 10 percent hemolysis resulted. Samples with increasingly finer particles caused 15.83 percent, 32.48 percent, and 40 percent hemolysis, respectively. Unfortunately, no surface area values were determined for this sample at this point.

In these data, no relationship between surface charge and degree of hemolysis is apparent. The surface charges of the asbestiform and acicular grunerite were found to be identical, yet their hemolytic activities were extremely different. On the other hand, the semiasbestiform and acicular cummingtonite had different surface charges but exhibited similar hemolytic activities.

Cytotoxicity

Cytotoxic to CHO was determined by counting the numbers of clones surviving after exposure to test substances as compared to the number of clones in the untreated

cultures. Asbestiform grunerite of surface area 4.13 m^2/g (FIGURE 1) was found to be the most toxic. At the dose of 0.05 mg/ml, less than 25 percent of the clones survived. At higher doses of 0.1 and 0.2 mg/ml, only 5 percent and 1 percent of the clones survived, respectively. Acicular grunerite of comparable surface area 2.82 m^2/g (Sample A, FIGURE 2) was nontoxic even at 0.50 mg/ml, a ten-times-higher dose. Acicular grunerite, when ground further (Sample B, FIGURE 3), was also nontoxic at the dose of 0.50 mg/ml. Samples with still finer particles (Sample C, FIGURE 4) were slightly toxic at 0.40 and 0.50 mg/ml, showing only 75 percent and 45 percent survival, respectively. In the samples with the most fine particles (Sample D, FIGURE 5), toxicity was observed at the 0.20 mg/ml dose. Seventy-five percent of the clones survived at 0.20 mg/ml, 55 percent at 0.30, 40 percent at 0.40, and 35 percent at 0.50 mg/ml. No significant toxicity was observed at lower concentrations of 0.05 and 0.10 mg/ml.

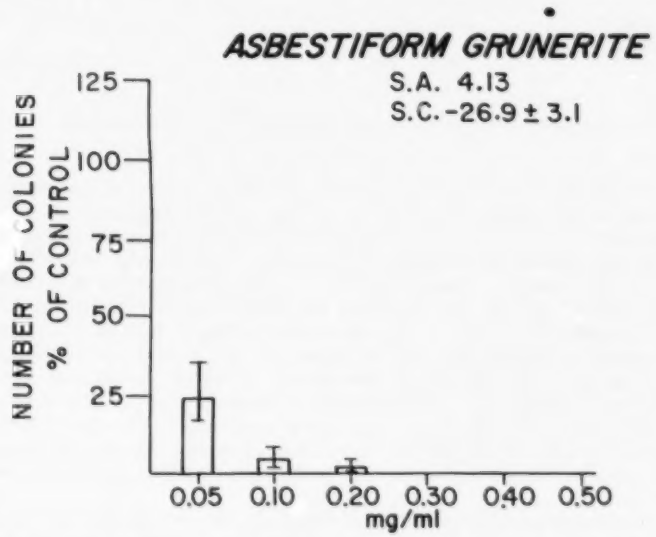

FIGURE 1. Surviving CHO clones (expressed as percent of untreated controls) following exposure to various doses of asbestiform grunerite, UICC amosite, (S.A. 4.13 m^2/g).

The levels of cytotoxicity caused by semiasbestiform and acicular varieties of cummingtonite were compared. Semiasbestiform of surface area 3.88 m^2/g was relatively more toxic than the acicular variety of comparable surface area 3.76 m^2/g (FIGURES 6 and 7). At the doses of 0.05 and 0.10 mg/ml, 85 percent and 70 percent of the clones survived, respectively, when exposed to the semiasbestiform variety. No toxicity was observed at these doses with exposure to the acicular variety. At higher concentrations, no significant difference in cytotoxicity was observed; however, in both instances the cytotoxicity levels were dose-dependent. Similar levels of cytotoxicity were caused by the semiasbestiform samples of surface areas 1.61 m^2/g and 3.88 m^2/g (FIGURES 8 and 6). The acicular variety of surface area 2.45 m^2/g (FIGURE 9) was relatively less toxic, however. No toxicity was obvious up to 0.30 mg/ml. At 0.40 and

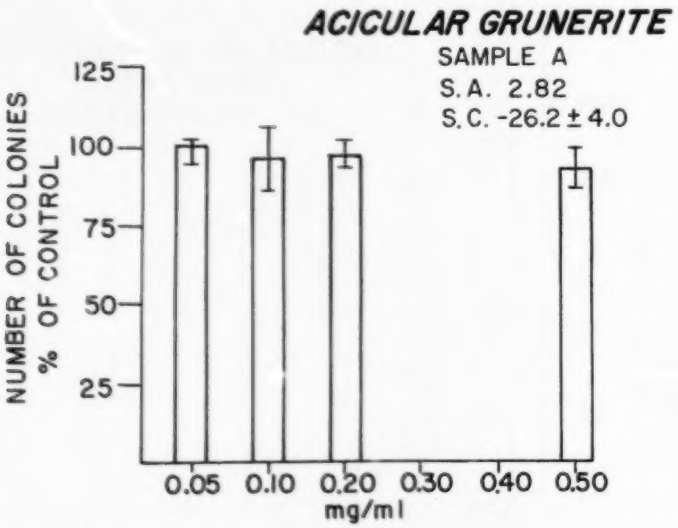

FIGURE 2. Surviving CHO clones (expressed as percent of untreated controls) following exposure to various doses of acicular grunerite, (S.A. 2.82 m^2/g, Sample A).

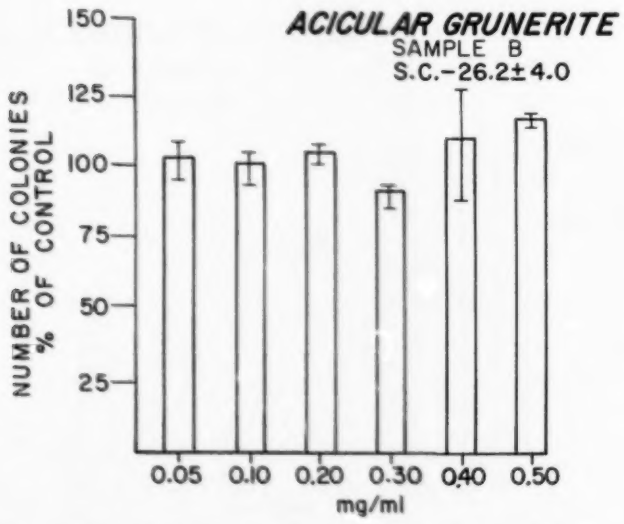

FIGURE 3. Surviving CHO clones (expressed as percent of untreated controls) following exposure to various doses of acicular grunerite (Sample B).

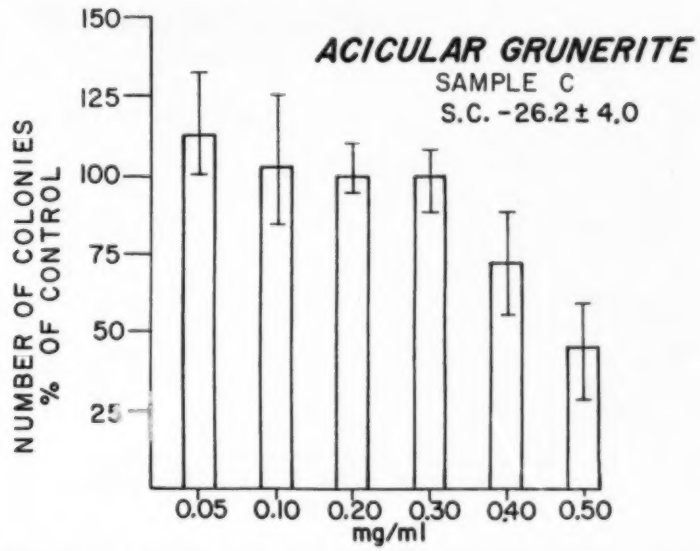

FIGURE 4. Surviving CHO clones (expressed as percent of untreated controls) following exposure to various doses of acicular grunerite (Sample C).

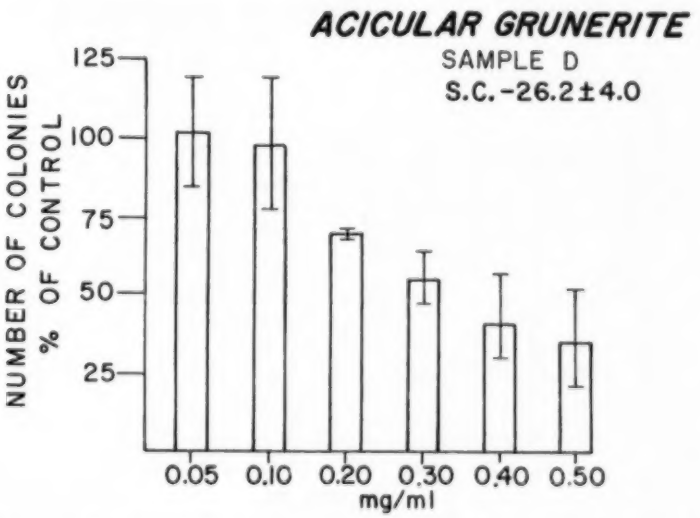

FIGURE 5. Surviving CHO clones (expressed as percent of untreated controls) following exposure to various doses of acicular grunerite (Sample D.)

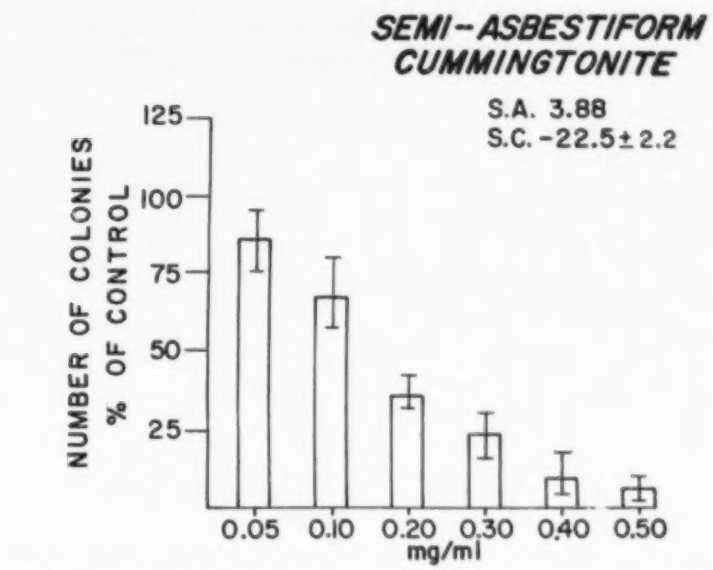

FIGURE 6. Surviving CHO clones (expressed as percent of untreated controls) following exposure to various doses of semiasbestiform cummingtonite, (S.A. 3.88 m^2/g).

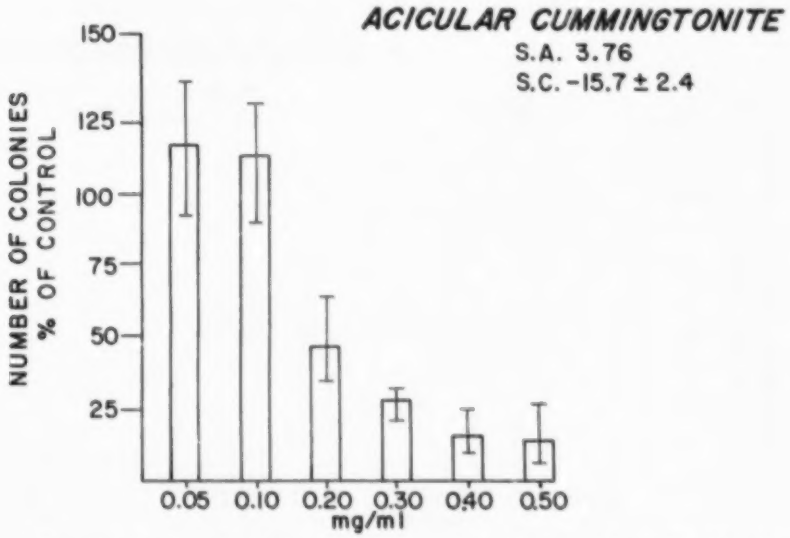

FIGURE 7. Surviving CHO clones (expressed as percent of untreated controls) following exposure to various doses of acicular cummingtonite (S.A. 3.76 m^2/g).

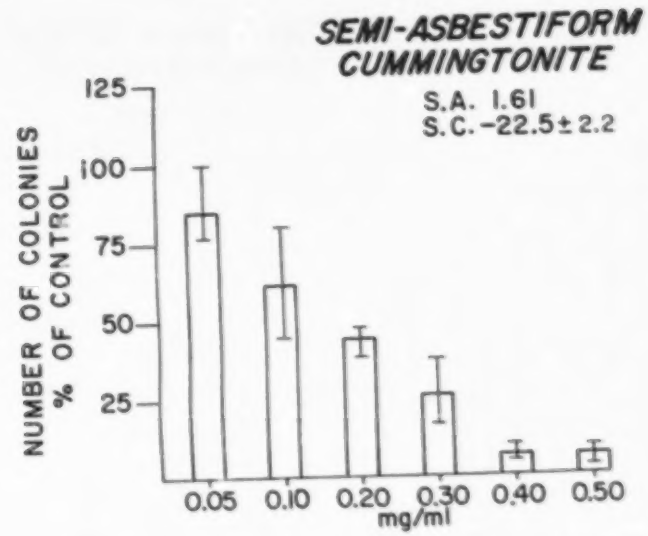

FIGURE 8. Surviving CHO clones (expressed as percent of untreated controls) following exposure to various doses of semiasbestiform cummingtonite (S.A. 1.61 m^2/g).

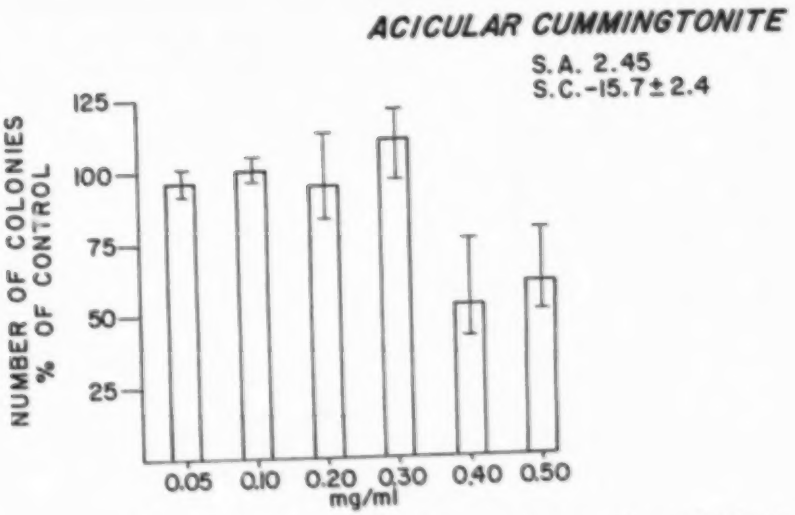

FIGURE 9. Surviving CHO clones (expressed as percent of untreated controls) following exposure to various doses of acicular cummingtonite (S.A. 2.45 m^2/g).

0.50 mg/ml, more than 50 percent of the clones survived. The semiasbestiform sample of surface area 1.21 (FIGURE 10) was slightly less toxic than the two other samples (surface areas 3.88 and 1.61 m^2/g). The acicular cummingtonite of surface area 0.82 m^2/g (FIGURE 11) was nontoxic even at a high dose of 0.5 mg/ml.

The relationship between surface area and toxicity was not quite obvious in the semiasbestiform samples. The cytotoxicity caused by acicular cummingtonite, however, was found to be related to surface area. Samples of higher surface area were more toxic than those of lower surface area.

In this study, there was no correlation between surface charge and cytotoxicity to CHO cells. Asbestiform grunerite and acicular grunerite with similar surface charges (−26.9 and −26.2 mV, respectively) caused cytotoxicity of extreme values. The

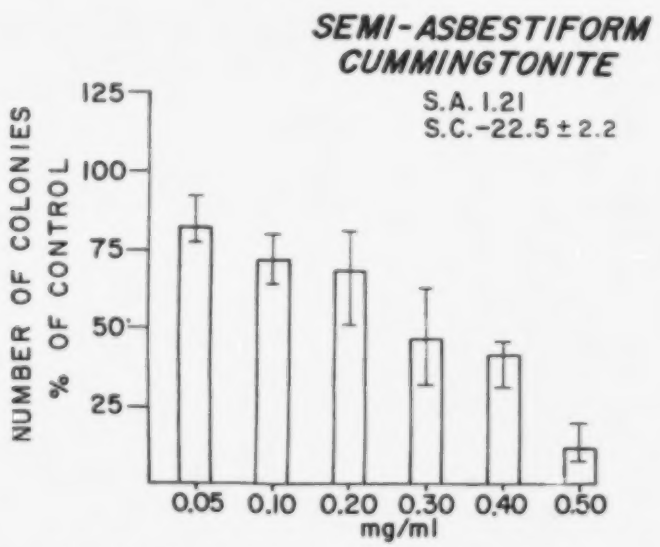

FIGURE 10. Surviving CHO clones (expressed as percent of untreated controls) following exposure to various doses of semiasbestiform cummingtonite (S.A. 1.21 m^2/g).

semiasbestiform and acicular cummingtonite were of different surface charge; the cytotoxicity caused by these samples was, however, found to be similar.

DISCUSSION

The asbestiform development of chain silicates is a continuous process depending on the earth's cooling history, temperature, pressure, presence of water, and other physical conditions. Since these conditions may be different in different geological regions, it is quite common to find silicates of similar chemical series in various developmental stages of crystallization habits. Some chain silicates may consist entirely of perfect long fibers with high tensile strength, some may consist of acicular,

noticeably weaker and brittle crystals, and yet others may consist of a mixture of high-tensile strength and acicular crystals with variable structural faults and surface defects.[8]

The chain silicate asbestos types exploited for commercial use are of the first category (perfect, long fibers of high-tensile strength). Epidemiological studies have clearly revealed that exposure to commercial asbestos is hazardous to human health. *In vitro* cytotoxicity studies and hemolysis studies have also shown that commercial asbestos is cytotoxic and hemolytic. In this study, we investigated the cytotoxicity and hemolytic activity of four samples of cummingtonite-grunerite series in four different crystallization habits.

The complexity of interaction of minerals with biological systems has been suggested by several investigators. The significance of surface area in hemolysis was

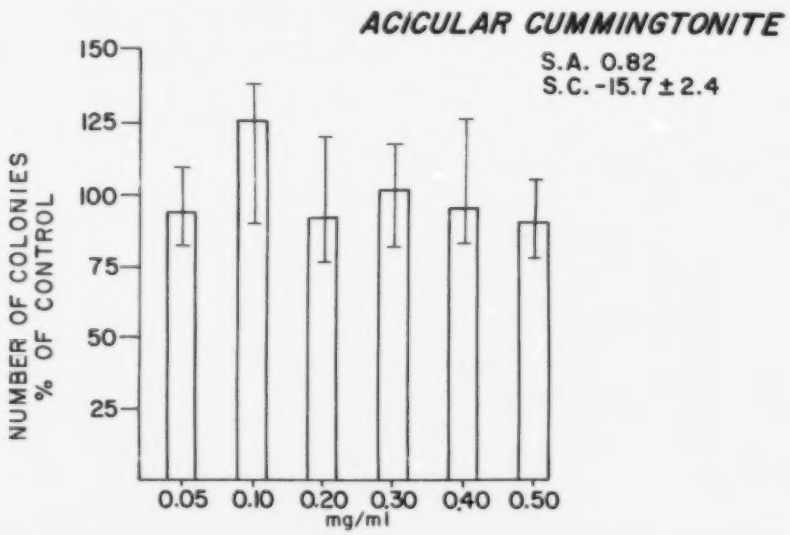

FIGURE 11. Surviving CHO clones (expressed as percent of untreated controls) following exposure to various doses of acicular cummingtonite (S.A. 0.82 m^2/g).

indicated by Harrington *et al.*,[1] who reported that the degree of hemolysis caused by several UICC asbestos samples was proportional to surface area. Light and Wei,[3,4] on the other hand, demonstrated that, at a dose compensated to provide an equal amount of surface area, the hemolysis caused by UICC asbestos samples was proportional to surface charge. The importance of Mg ions has also been investigated. When Mg was removed from UICC chrysotile by acid wash, it became inert,[1,5,6] pointing out the role of Mg in hemolysis; however, the investigators were unable to document this relationship with other UICC amphiboles. For these reasons, our samples were carefully characterized for surface area, surface charge, and chemical content. A careful evaluation was undertaken to determine whether there is indeed a relationship between the two biological systems studied and the crystallization habits.

The initial comparision between the four samples of different crystallization habits

was made at comparable surface areas of 4.13, 3.88, 3.76, and 2.82 m^2/g of UICC amosite, semiasbestiform cummingtonite, acicular cummingtonite, and acicular grunerite, respectively. The data indicate that hemolysis of sheep erythrocytes and cytotoxicity to CHO cells was inversely proportional to the degree of development of asbestos character in the minerals. Amosite from South Africa with high-tensile strength and practically no surface defects was most hemolytic and cytotoxic. The other three samples (semiasbestiform cummingtonite, acicular cummingtonite, and acicular grunerite) with limited or no asbestos character in increasing order were hemolytic and cytotoxic in decreasing order, the most nonasbestiform mineral being totally inert. The degree of hemolysis caused by UICC amosite is in agreement with the reports of other investigators.[1,2,5]

The relationship of hemolysis and cytotoxicity to particle size became clear when several samples of acicular grunerite were compared. Acicular grunerite of relatively low surface area (2.82 m^2/g) was totally inert; when the samples were then ground further, they were hemolytic and cytotoxic. This relationship was not quite apparent in the levels of hemolysis caused by semiasbestiform cummingtonite and acicular cummingtonite; it was, however, obvious in the cytotoxicity assay. Unfortunately, surface areas of the finer acicular grunerite samples were unavailable. Hence, conclusive correlation cannot be made between surface area and biological activities.

No relationship between surface charge and hemolysis (as well as cytotoxicity) was apparent. The complexicity of surface charge in biological systems has been indicated by Light and Wei.[3,4] Surface charge is known to vary according to pH, ionic strength, and the amounts of serum or surfactant present in the system. The zeta potentials for these studies were obtained in Veronal-buffered solution at pH 7.4, rather than in distilled water at pH 7.4. The surface charge value for UICC amosite in Veronal buffer was -26 mV as compared to -58.5 mV reported by other investigators.[3] However, since Veronal buffer was used for hemolysis assay, it seemed appropriate to use this system for determinations. Moreover, the hemolysis and cytotoxicity assays used in this investigation involved the presence of hemoglobin and serum, respectively. The fate of surface charge of the particles after being exposed to the sheep erythrocytes and CHO cells was not calculated. More work will be required to establish the role of surface charge in these biological systems.

Analysis of these data indicate that crystallization habit plays an important role in hemolysis and cytotoxicity. Although asbestiform minerals are known to be hazardous (and demand significant attention), other minerals of semiasbestiform and acicular variety should not be ignored. It seems possible that, at higher doses and surface areas, the nonasbestiform minerals could also be hazardous to human health. More epidemiological and experimental research will be required to fully understand minerals and their effects in biological systems.

Summary

Four samples of cummingtonite-grunerite series in various crystallization habits were tested *in vitro*. The cytotoxicity to Chinese hamster ovary cells and hemolysis to sheep erythrocytes was inversely proportional to the structural faults and surface defects of the minerals. At a comparable surface area, asbestiform grunerite (UICC amosite), semi-asbestiform cummingtonite, acicular cummingtonite, and acicular grunerite were found to be cytotoxic and hemolytic in a decreasing order. The influence of particle size on hemolysis and cytotoxicity was observed with acicular grunerite. Although samples of relatively large particle size were found to be inert, samples of smaller particle size were cytotoxic as well as hemolytic. No apparent

relationship between surface charge and hemolysis as well as cytotoxicity was observed.

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